IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Millar, et al.
Appl. No.	:	10/428,310
Filed	:	May 2, 2003
For	:	TREATMENT OF METHYLATED NUCLEIC ACID
Examiner	:	Fredman, J.
Group Art Unit	:	1637

DECLARATION UNDER 37 CFR §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Dr John Robert Melki, declare and state:

1. I am Senior Principal Research Scientist at Human Genetic Signatures Pty Ltd., the assignee of the above-referenced patent application (***the Application***).

2. I am an expert in the field of molecular biology including DNA methylation. My Curriculum Vitae is enclosed (Exhibit A).

3. I am a designated inventor on 7 patents or pending patent applications, I have authored 11 peer reviewed scientific papers in the field of molecular biology and sodium bisulphite conversion of DNA, and have presented at more than 11 scientific meetings.

4. My research currently relates to the use of sodium bisulphite conversion of DNA to aid in the detection of microorganisms.

5. I am familiar with the Application and pending claims. I understand that the claims were rejected as obvious over Raizis et al. (Anal. Biochem. 226:161-166, 1995) in view of Olek et al. (WO 02/46452) and further in view of Clark et al. (Nucleic Acids Res. 22:2990-2997, 1994). I have reviewed the pending claims and these references, and herein describe experimental data supporting the unexpected results obtained using the claimed method.

6. I note that Clark et al. (Nucleic Acids Res. 22:2990-2997, 1994) has been cited by the Examiner. I worked in Associate Professor Sue Clark's laboratory for 8 years, during which time I attempted to improve the recovery of bisulphite converted DNA. During that work I carried out with Associate Professor Sue Clark, we were unable to substantially improve the recovery of DNA using the methods of Clark et al and others.

7. Human Genetic Signatures Pty Ltd (the assignee of the Application) has developed and sells a kit (MethylEasy™) for the treatment of methylated DNA in accordance with the invention disclosed and claimed in the Application.

8. To demonstrate the improvement of the invention of the Application, I carried out experiments to determine the effects of pH and temperature on the desulfonation step used in the bisulphate treatment of DNA. I designed experiments to determine the amount of DNA degradation when converting cytosine residues to uracil in DNA using Sodium Bisulphite in various conditions. The results of my experiments demonstrated the unexpected results obtained when the method is carried out using a desulfonation pH of between 10 and less than 12.5 at temperatures between 70°C and 95°C compared to the prior art desulfonation pH of 9 or 13 at temperatures around 37°C.

9. To test the affect of pH and temperature on the desulfonation step of the invention according to the Application, I carried out the following experiments and provide a summary of the data obtained.

10. Methods: 16 µg of Promega human genomic DNA was converted using sodium bisulphite solution as provided in the MethylEasy™ commercial kit in 8 independent reactions each containing 2 µg of DNA. The reactions were carried out at 55°C for 7.5 hours. All of the samples were pooled post incubation and mixed well and then re-aliquotted into 8 equal amounts into eight 2 ml Eppendorf tubes. I added 20 µg of glycogen (Roche) and 800 µl of MethylEasy™ reagent 4 to each tube, mixed well and added 1 ml of isopropanol to each tube and incubated at 4°C overnight.

11. All samples were then centrifuged at 4°C for 20 min, washed with 70% ethanol and resuspended in 200 µL of pH adjusted reagent 3, with the following pH values:

1) 8.06
2) 9.03
3) 10.02
4) 10.99
5) 12.02
6) 12.49
7) 13.00

12. 10 µl of each sample was desulphonated for 20 min at 65°C, 70°C, 80°C, 85°C, and 95°C and then 2 µl was added to the 1st round of a PCR amplification for the hmGST gene, as provided in the MethylEasy™ kit.

2x Promega mastermix	= 12.5 µl
Outer Primer 1	= 1.0 µl
Outer Primer 2	= 1.0 µl
Bisulphite converted DNA	= 2.0 µl
Nuclease Free Water	= 8.5 µl .
Total	= 25.0 µl

PCR conditions: (95°C x 3') x1; (95°C x 1', 50°C x 2', 72°C x 2') x30; (72°C x 5') x1

13. 1.5 µl of the above was added to the following pre-mix:

2x Promega mastermix	= 12.5 µl
Inner Primer 1	= 1.0 µl
Inner Primer 2	= 1.0 µl
Syto-9 intercalating dye	= 1.0 µl
Nuclease Free Water	= 8.0 µl .
Total	= 23.5 µl

14. The above PCR was run on a Corbett Rotor-Gene™ 6000 instrument using the following profile:

(95°C x 1') x1; (95°C x 5", 50°C x 1.5', 72°C x 1.5', 75°C x 1") x30

15. Data was collected and analysed at the 75°C time point to be free of any primer dimer fluorescence.

16. Using a standard delta Ct analysis, relative expression was determined and was plotted and the results set out in (Exhibit B).

17. The data shows that desulphonation at pH 13, under all temperatures tested, no DNA amplification was detected. This clearly demonstrates that the typical desulphonation pH used in the prior art does not work in accordance with the invention in the Application.

18. From the results obtained, I found that desulphonation using a pH range of 10 to approximately 12.5 at temperature at 65°C was not as good at preserving the original DNA compared with desulphonation using temperatures between 70°C and 95°C at the same pH range. In my experience, temperatures higher than 95°C can result in unwanted destruction of DNA so are not generally used when handling DNA.

19. From the results obtained, I found that desulphonation using a pH range of 10 to approximately 12.5 was far better at preserving the original DNA than desulphonation using a higher or lower pH.

20. To determine whether there may have been a "buffer effect" in the desulphonation reaction carried out in accordance with the invention of the Application, experiments were carried out at the same pH using either NaOH in a buffer (TE) or using NaOH in water. DNA yields between the same pH were compared.

21. 2 x 33.3 ng of DNA (Promega human DNA) was converted with sodium bisulphite using standard MethylEasy™ conditions for 4 hours at 80°C. The converted DNA was desalted and resuspended in either water or in buffer that had been pH adjusted to 10.0 for desulphonation reaction. Results are shown in Exhibit C.

22. It can be clearly seen from the results in Exhibit C that buffer had no effect on the desulphonation reaction. Only a pH range of 10 to approximately 12.5 is required for the invention of the Application to be carried out, irrespective of the diluent used.

23. I note Raizis et al. (*Anal. Biochem.* 226:161-166, 1995) uses room temperature and pH 13 (0.2 M NaOH) for their desulphonation step. In my experience, a desulphonation step at room temperature and pH 13 using the other conditions according to the invention of the Application results in high losses of DNA.

24. I understand that Clark et al. (*Nucleic Acids Res.* **22**:2990-2997, 1994)) is part of the basis of an obvious rejection of the claims of the Application. I note that it is stated that Clark et al. expressly teaches that each step of the protocol should be optimised. In a subsequent Clark publication, Grunau, Clark and Rosenthal (*Nucleic Acids Res.* 29: No 13, 2001) a copy of which is enclosed (Exhibit D), used 0.3M NaOH in the desulphation step (pH of >13) at 37°C. The authors clearly state under the section entitled "*Desulphonation*" in the fourth column that sodium hydroxide concentration of less than 0.3M (and therefore lower pH) will yield incomplete desulphonation (data not shown). The authors stated that the desulphonation step used had previously been optimised (Reference 16, Clark and Fromer 1997) which uses 0.3M NaOH. I note that the 'optimized method' of Clark and others was found to degrade 84-96% of the DNA.

25. From my experience with detecting methylation in DNA, the MethylEasy™ kit made in the accordance with the invention of the Application out performs other methods developed, published and used before this invention was developed by Human Genetic Signatures. The invention of the Application allows vastly improved recovery of DNA compared with the prior art methods. Furthermore, the invention of the Application allows the treatment of smaller amounts of starting DNA compared with prior art methods thus providing more sensitive analysis of methylation of DNA.

26. I have compared the performance of the commercial bisulphite treatment kits sold by Chemicon (CpGENOME) and Zymo Research (EZ DNA). To my knowledge, these two kits are based on prior published methods of bisulphite treatment of DNA and were the only kits available for sale at the time the MethylEasy™ kit was released for sale in March 2004. In my experience, the MethylEasy™ kit out performed both kits. The MethylEasy™ kit provided far greater recovery of DNA and could be used with lower amounts of starting DNA compared with the two competing kits. I have received feedback from other researchers who have also carried out this type of comparison and have been informed by the other researchers that the MethylEasy™ kit was found in their laboratories to be at least ten times more sensitive compared with performance of competing commercial kits.

27. The competing kits that were available for sale at the time of the launch of the MethylEasy™ kit were all based on the prior art bisulphite treatment (Clark et al, cited in present Office Action). There is no significant difference in the buffers used in the DNA denaturation or incubation in sodium bisulphite steps of the invention and the kits tested. Thus, the difference between the results obtained with the present invention and the results obtained with the other kits tested are a consequence of performing the desulphonation at elevated temperatures and a pH range of between 10 and less than 12.5 rather than differences between the buffers.

28. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

By: John Melki Date: 29th March 2007

Dr John Robert Melki

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Exhibit A

RESUME OF JOHN ROBERT MELKI

PERSONAL DETAILS

Name: John Robert Melki

Address: 51 Fontainebleau St
Sans Souci, 2219.
Sydney, NSW, AUSTRALIA

Phone: Cellular: +61 411 339 471
business: +61 2 9870 7116
Fax: +61 2 9888 7774
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EDUCATION DETAILS

1995-2000 **PhD Faculty of Medicine**
University of Sydney, Australia
Thesis: "DNA Methylation in Leukaemia"
This thesis was awarded the Peter Bancroft Prize (Faculty of Medicine) for 2000.

1991-1994 **BSc(Hons, 1st class in Molecular Genetics)**
University of New South Wales, Australia
Thesis: "Competitive PCR as a method to measure expression levels and to estimate genome size" Mark: 94

1985-1990 **Higher School Certificate**
St. Andrews Cathedral School, Sydney, Australia.
93rd Percentile

EMPLOYMENT HISTORY

2003 - current **Scientist**
Human Genetic Signatures Pty Ltd

- I began with this start-up biotechnology company when it was still in its infancy in 2003. I am involved in day-to-day experimental work, mostly involving PCR and microarrays and have submitted 4 patent applications that are in various stages of advancement in the patent system.
- I am a member of the Product Development team that has since brought 2 products and 2 services to the marketplace. I was involved in most aspects of bringing the kits to market, including experimental work-up, packaging design/production, website development and manual writing. Part of this role

involves traveling interstate and internationally to meet with existing and potential customers. I am also a member of the management team, that assesses the potential of upcoming products.

2002 - 2003 **Senior Research Officer**
Sydney Cancer Centre

- Maintained responsibilities from Research officer position, and took on additional duties of overseeing the laboratory, and supervision of a research assistant. Responsibilities also included establishing and maintaining external collaborations, as well as maintaining all computers, printers and networks.
- Established the Chromatin Immunoprecipitation assay (ChIP) for the lab.

2000 – 2002 **Research Officer**
Sydney Cancer Centre

- Characterisation of CpG Methylation patterns in Human Mammary Epithelial Cells and other model systems. This work involves establishing the methylation patterns in very few cells using laser capture technology.
- Differential expression analysis using array technology (Clontech arrays, analysed with atlasimage and genespring software).
- DNA Methyltransferase expression studies in various systems (TaqMan), as well as validation of array results.

1994 – 1995 **Research Assistant**
Kanematsu Laboratories

- Tissue Culture
- CAT/SAT assays

1993 – 1994 **Summer Scholar**
CSIRO Biomolecular Engineering

PUBLICATIONS

1. Noble, J. R., Zhong, Z. H., Neumann, A. A., Melki, J. R., Clark, S. J. and Reddel, R. R. **Alterations in the p16(INK4a) and p53 tumor suppressor genes of hTERT-immortalized human fibroblasts.** Oncogene, 23:3116-21., 2004

2. Melki, J. R. and Clark, S. J. DNA methylation changes in leukaemia. Semin Cancer Biol, 12: 347-357., 2002.

3. Clark, S. J. and Melki, J. DNA methylation and gene silencing in cancer: which is the guilty party? Oncogene, 21: 5380-5387., 2002.

4. Millar, D. S., Warnecke, P. M., Melki, J. R., and Clark, S. J. Methylation sequencing from limiting DNA: embryonic, fixed, and microdissected cells. Methods, 27: 108-113., 2002.

5. Song, J. Z., Stirzaker, C., Harrison, J., Melki, J. R., and Clark, S. J. Hypermethylation trigger of the glutathione-S-transferase gene (GSTP1) in prostate cancer cells. Oncogene, 21: 1048-1061., 2002.

6. Melki, J. R., Vincent, P. C., Brown, R. D., and Clark, S. J. Hypermethylation of E-cadherin in leukemia. Blood, 95: 3208-3213., 2000.

7. Melki, J. R., Vincent, P. C., and Clark, S. J. Concurrent DNA hypermethylation of multiple genes in acute myeloid leukemia. Cancer Res, 59: 3730-3740., 1999.

8. Melki, J. R., Vincent, P. C., and Clark, S. J. Cancer-specific region of hypermethylation identified within the HIC1 putative tumour suppressor gene in acute myeloid leukaemia. Leukemia, 13: 877-883., 1999.

9. Huschtscha, L. I., Noble, J. R., Neumann, A. A., Moy, E. L., Barry, P., Melki, J. R., Clark, S. J., and Reddel, R. R. Loss of p16INK4 expression by methylation is associated with lifespan extension of human mammary epithelial cells. Cancer Res, 58: 3508-3512., 1998.

10. Melki, J. R., Warnecke, P., Vincent, P. C., and Clark, S. J. Increased DNA methyltransferase expression in leukaemia. Leukemia, 12: 311-316., 1998.

11. Warnecke, P. M., Stirzaker, C., Melki, J. R., Millar, D. S., Paul, C. L., and Clark, S. J. Detection and measurement of PCR bias in quantitative methylation analysis of bisulphite-treated DNA. Nucleic Acids Res, 25: 4422-4426., 1997.

INVITED TALKS

1. **Finding the methylation trigger in cancer.** Methylation Matters Symposium, Sydney. November 2002.
2. **DNA Methylation in Leukaemia.** CSIRO Molecular Science, Sydney. September 1998.
3. **DNA Methylation in Malignancy.** University of New South Wales, Dept of Biochemistry. September 1998.
4. **DNA Methylation in Malignant Disease.** Haematology Society of Australia, Ritz Carlton Hotel, Sydney, September 1996.
5. **DNA Methyltransferase Expression in Leukaemia.** Medical Faculty Symposia, RPAH, at Wollongong, NSW, July 1996.

CONFERENCE PRESENTATIONS (POSTERS)

1. Melki, J. Vincent, P. & S.J. Clark. Understanding the mechanism of CpG island methylation in malignancy. Gordon conference on Cancer Genetics and Epigenetics, Feb, 2000, Ventura, CA
2. Paul C. Melki J & Clark S, Improved quantitative sequencing for methylated DNA. 19th. Annual Genome Conference, Lorne, February, 1998.
3. Melki, J. Vincent, P. and Clark, S. DNA Methyltransferase Expression in Leukaemia. 26th Annual Meeting of the International Society for Experimental Hematology , Cannes, France, August, 1997.
4. Melki, J. Vincent, P. and Clark, S. DNA Methyltransferase Expression in Leukaemia. FASEB Summer Research conference, Biological Methylation, Vermont, USA, 1997.
5. Melki, J. Vincent, P. and Clark, S. DNA Methylation in Leukaemia. 8th Lorne Cancer Conference, 1996.
6. Melki, J.R and Mackinlay, A.G. Competitive PCR to estimate genome size. Lorne Genome Conference, February, 1995.

AWARDS AND GRANTS

2000 University of Sydney Faculty of Medicine Peter Bancroft Prize (For my PhD thesis).

1998-2001 Anthony Roth Memorial Trust Grant: Detection of Hypermethylated DNA provides an early marker to monitor Leukaemia. (Investigators: S. Clark, J. Melki, P. Vincent)

1995-1999 Anthony Rothe PhD Scholarship

1995-1998 Australian Postgraduate Research Award (conferred, but not accepted)

1993-1994 Summer vacation scholarship - CSIRO Division of Biomolecular Engineering

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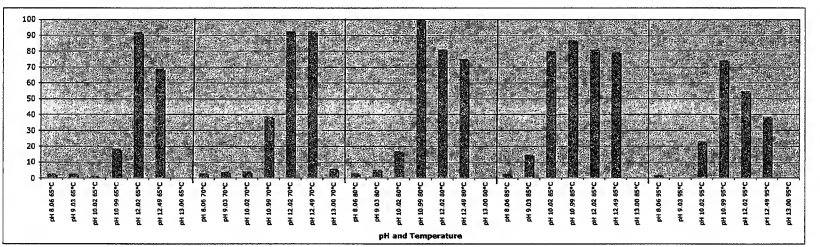
Exhibit B



y-axis – Relative Signal

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Exhibit C